0026-895X/02/6101-201–213$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2002 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 61:201–213, 2002

Vol. 61, No. 1
941/953855
Printed in U.S.A.

Molecular Basis of Voltage-Dependent Potassium Currents in Porcine Granulosa Cells

DIANE E. MASON, KATHY E. MITCHELL, YAN LI, MELISSA R. FINLEY, and LISA C. FREEMAN

Departments of Anatomy and Physiology (K.E.M., Y.L., M.R.F., L.C.F.) and Clinical Sciences (D.E.M.), College of Veterinary Medicine, Kansas State University, Manhattan, Kansas

Received March 9, 2001; accepted July 20, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The major objective of this study was to elucidate the molecular bases for K^+ current diversity in porcine granulosa cells (GC). Two delayed rectifier K^+ currents with distinct electrophysiological and pharmacological properties were recorded from porcine GC by using whole-cell patch clamp: 1) a slowly activating, noninactivating current (I_{Ks}) antagonized by clofilium, 293B, L-735,821, and L-768,673; and 2) an ultrarapidly activating, slowly inactivating current (I_{Kur}) antagonized completely by clofilium and 4-aminopyridine and partially by tetraethylammonium, charybdotoxin, dendrotoxin, and kaliotoxin. The molecular identity of the K^+ channel genes underlying I_{Ks} and I_{Kur} was examined using reverse transcription-polymerase chain reaction and immunoblotting to detect K^+ channel transcripts and proteins. We found that GC could express multiple voltage-dependent K^+ (Kv) channel subunits, including KCNQ1, KCNE1, Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kvβ1.3, and Kvβ2. Coimmunoprecipitation was used to establish the hetero-oligomeric nature of granulosa cell Kv channels. KCNE1 and KCNQ1 were coassociated in GC, and their expression coincided with the expression of I_{Ks}. Extensive coassociation of the various Kv α- and β-subunits was also documented, suggesting that the diverse electrophysiological and pharmacological properties of I_{Kur} currents may reflect variation in the composition and stoichiometry of the channel assemblies, as well as differences in post-translational modification of contributing Kv channel subunits. Our findings provide an essential background for experimental definition of granulosa K^+ channel function(s). It will be critical to define the functional roles of specific GC K^+ channels, because these proteins may represent either novel targets for assisted reproduction or potential sites of drug toxicity.

Granulosa cells (GC) surround the oocyte within the ovarian follicle and play an essential role in creating the conditions required for follicular development, ovulation, fertilization, and implantation (Salustri et al., 1993). During folliculogenesis, GC undergo a series of mitotic divisions (proliferation) then acquire gonadotropin receptors and enhanced steroidogenic activity (differentiation). Autocrine-paracrine and endocrine regulation of the granulosa cell maturation have been extensively investigated and much is known about the specific roles of various growth factors, hormones, transmembrane receptors, and second messengers (Steele and Leung, 1993). In contrast, the functional significance of voltage-dependent ion channels in GC is far from understood.

It has been reported that GC can generate action potentials (Mealing et al., 1994), and indirect evidence suggests that modulation of granulosa cell electrical activity may provide a means to regulate cell function (Mattioli et al., 1990, 1991, 1993; Kusaka et al., 1993). For example, granulosa cell depolarization has been described as a consistent feature of oocyte maturation in different experimental systems (Mattioli et al., 1990). Voltage-gated potassium currents with distinct electrophysiological and pharmacological properties have been described in both acutely isolated and cultured GC, and have been shown to regulate granulosa cell resting

These studies were supported by National Institutes of Heath grants HD34235 and HD36002 (to L.F.). D.E.M. and K.E.M. contributed equally to this study.

ABBREVIATIONS: GC, granulosa cells; Kv1, voltage-gated K^+ channel subfamily 1 member; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; FSH, follicle-stimulating hormone; MK-499, [(+)-*N*-{1'-(6-cyano-1,2,3,4-tetrahydro-2(*R*)-naphthalenyl)-3,4-dihydro-4(*R*)-hydroxyspiro(2*H*-1-benzopyran-2,4'-piperidin)-6-yl]methanesulfonamide]; 293B, 4-(*N*-ethylsulfonyl-*N*-methylamino)-3-hydroxy-2,2-dimethylchromane; LY97241, 4-ethyl-*N*-heptyl-4-nitrobenzenebutanamine ethanedioic acid; L735,821, (*R*)-2(ethylene-2,4-dichlorophenyl)-*N*-[2-oxo-5-phenyl-1-methyl-2,3-dihydro-1*H*-benzol[*e*][1,4]diazepin-3-yl]acetamide; L768,673, (*R*)-2-(2,4-trifluoromethylphenyl)-*N*-[2-oxo-5-phenyl-1-(2,2,2-trifluoroethyl)-2,3-dihydro-1*H*-benzol[*e*][1,4]diazepin-3-yl]-acetamide; HEK, human embryonic kidney; HERG, human *ether-a-go-go*-related gene; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; I_{Ks}, slow delayed rectifier K^+ current; I_{Kur}, ultra-rapid delayed rectifier K^+ current; bp, base pair; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; PNGase-F, peptide-*N*-glycosidase F; 4-AP, 4-aminopyridine; TEA, tetraethylammonium; CTX, charybdotoxin; DTX, dendrotoxin; KTX, kaliotoxin; MTX, margatoxin; CHO, Chinese hamster ovary; RT, reverse transcription.

membrane potential (Mattioli et al., 1991, 1993; Kusaka et al., 1993). Delayed rectifier currents with both slow and rapid activation and inactivation kinetics have been described in porcine GC (Mattioli et al., 1991, 1993; Kusaka et al., 1993). However, the molecular correlates of these currents have not been identified.

The major objective of this study was to elucidate the molecular bases for K^+ current diversity in porcine GC. Specific antibodies were used to demonstrate directly the existence in GC of Kv1 (KCNA) and KCNQ1 (KvLQT1) channel proteins, as well as Kvβ (KCNAB) and KCNE1 (minK or IsK) auxiliary subunits. Coimmunoprecipitation was used to establish the hetero-oligomeric nature of granulosa cell Kv channels. Whole-cell patch-clamp techniques were used to record granulosa cell K^+ currents and to document the specific effects of a variety of K^+ channel antagonists.

We report not only that a variety of voltage-gated K^+ channel α- (pore-forming) and β- (accessory) subunits are present in freshly isolated GC but also that their expression is temporally regulated as GC spontaneously differentiate (luteinize) in culture. Our data suggest that the diverse electrophysiological and pharmacological properties of native granulosa cell K^+ currents, described here and elsewhere (Mattioli et al., 1991, 1993; Kusaka et al., 1993), may reflect variation in the composition and stoichiometry of the hetero-oligomeric channel complexes, as well as differences in post-translational modification of contributing channel subunits. The documented potential for dynamic interchange of various associated K^+ channel subunits as a function of the cells' metabolic status suggests that these ion channels may participate in control of granulosa cell proliferation or differentiation.

Materials and Methods

Reagents. Cell culture media, supplements, and sera were obtained from Invitrogen (Carlsbad, CA), unless stated otherwise. Chemicals were obtained from Sigma Chemical (St. Louis, MO), unless stated otherwise. Regular pork insulin and LY-97241 were obtained from Eli Lilly (Indianapolis, IN). Margatoxin and charybdotoxin were obtained from Alomone Laboratories (Jerusalem, Israel). Anti-phosphotyrosine cocktail (PY-*Plus*-HRP) was purchased from Zymed Laboratories (South San Francisco, CA). Polyclonal rabbit antibodies directed against Kv1.1, Kv1.2, Kv1.3, Kv1.5, and Kv1.6 α-subunits were purchased from Alomone Laboratories. Mouse monoclonal antibodies directed against Kv1.1, -1.2, -1.4, and -1.5 were obtained from Upstate Biotechnology (Lake Placid, NY). Primary antibodies directed against HERG were a gift from J. Nerbonne (Pond et al., 2000). Primary antibody directed against KvLQT1 was a gift from D. Roden and S. Kupershmidt (Vanderbilt University, Nashville, TN). Primary antibody directed against minK was a gift from R. Dumaine (Masonic Medical Research Laboratory, Utica, NY). Primary antibody directed against Kvβ1.3 was a gift from M. Tamkun (Colorado State University, Ft. Collins, CO). Nitrocellulose membranes (Hybond ECL), secondary antibodies, chemiluminescence reagent (ECL), and film (Hyperfilm ECL) were obtained from Amersham Biosciences, Inc. (Piscataway, NJ). Porcine FSH was a gift from the National Hormone and Pituitary Program. Compound 293B was a gift from Aventis (Frankfurt, Germany). (−)-(3*R*,4*S*)-293B was obtained from Procter & Gamble (Cincinnati, OH). Compounds MK-499, L-735,821, and L-768,673 were obtained from Merck Research Laboratories (Westpoint, PA). HEK-293 cells stably transfected with HERG were provided by Z. Zhou and C. T. January (University of Wisconsin, Madison, WI). HERG cDNA was obtained from Robert Kass (Columbia University, New York, NY) Primers for non-nested PCR of KCNE1 (~170 bp) were supplied by M. Scofield (Creighton University, Omaha, NE).

Granulosa Cell Isolation and Culture. GC were isolated from the ovaries of mature swine by using techniques described in detail previously (Barano and Hammond, 1985). Briefly, small (1–3 mm in diameter) to medium (4–6 mm in diameter) follicles were aspirated by hand with a 19-gauge needle attached to a 10-ml syringe. GC were separated from follicular fluid by centrifugation at 500*g* for 5 min. Cells were washed twice with a 1:1 mixture of Ham's F-10 nutrient medium and Dulbecco's modified Eagle's medium (DMEM) containing HEPES (25 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). For RNA isolation or membrane protein preparation, the granulosa cell pellet was washed again with phosphate-buffered saline, flash frozen in liquid nitrogen, and kept at −80°C.

The basic medium for short-term primary culture of GC consisted of 1:1 DMEM/Ham's F-10 supplemented with fetal bovine serum (10%), penicillin (50 U/ml), streptomycin (50 μg/ml), Gentamicin (57 ng/ml), and amphotericin (2.5 μg/ml). Freshly isolated GC were plated on collagen-coated 24-well plates at a density of 2 to 3 × 10^5 cells, and cultured at 37°C in humidified atmosphere containing 5% CO_2. Culture media were changed 16 to 20 h after plating, and replaced at 24-h intervals thereafter. In one series of experiments, GC were incubated for 4 h in methionine-free DMEM containing 100 μCi/ml [^{35}S]methionine (PerkinElmer Life Sciences, Boston, MA) before harvest.

Electrophysiological Recordings. Membrane currents were recorded using standard patch-clamp procedures in the whole-cell configuration. Voltage-clamp protocols and solutions for measuring slow (I_{Ks}) and ultrarapid (I_{Kur}) cardiac delayed rectifier currents under whole-cell recording conditions have been described in detail previously (Arena and Kass, 1988; Nattel et al., 1999); similar protocols were used to elicit granulosa cell currents. Briefly, recording pipettes were pulled to resistances of 2.5 to 6 MΩ when filled with intracellular (pipette) solution containing 110 mM potassium aspartate, 1 mM $MgCl_2$, 11 mM EGTA, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM K_2ATP, pH 7.3, attained by addition of 1 N KOH to bring the final potassium concentration to 140 mM. Extracellular recording (bath) solution consisted of 132 mM NaCl; 1.2 mM $MgCl_2$; 1 mM $CaCl_2$; 5 mM glucose; 0.0, 0.5, or 4.8 mM KCl; 10 mM HEPES, pH adjusted to 7.4 with NaOH. The reference electrode was an Ag/AgCl half-cell immersed in the pipette solution and connected to the bath via a 3 M KCl-agar salt bridge. Tip potentials were zeroed before seal formation. All recordings were performed at room temperature (22–24°C) from a Plexiglas chamber mounted on an inverted microscope (Nikon Diaphot 300; Nikon, Tokyo, Japan). Data acquisition and analysis were accomplished using an IBM compatible computer interfaced to an Axopatch 200-A amplifier driven by pClamp software (Axon Instruments, Union City, CA). The standard voltage-clamp protocol for activation of I_{Ks} consisted of a series of 1- to 4-s depolarizing test pulses from a holding potential of −40 mV to test potentials (V_t) ranging from −40 to +60 mV at an interpulse interval of 14 s. I_{Kur} was elicited from holding potentials of either −80 to −40 mV by 80- to 1000-ms depolarizing test pulses to V_t between −40 and +60 mV.

The resting membrane potential of freshly isolated porcine GC averaged −38.4 ± 4.3 (n = 8). Granulosa cell membrane capacitance, calculated as the time integral of the capacitive response to a 5-mV hyperpolarizing step from a −40-mV holding potential was 16.1 ± 2.8 pF (n = 8). Series resistance, estimated by dividing the time constant of the decay phase of the uncompensated capacitive transient by the calculated membrane capacitance, was 2.6 ± 0.9 MΩ and was electrically compensated to minimize the duration of the capacity transient (>90%). Peak currents used to derive activation curves did not exceed 300 pA; therefore, the voltage errors associated with uncompensated series resistance never exceeded 1 mV.

I_{Kur} was measured as the current level at the end of a depolarizing pulse relative to the zero current level. I_{Ks} amplitude was similarly determined from the amplitudes of the tail currents recorded on

return to the holding potential. The voltage dependence of channel activation was determined by calculating normalized peak conductance values from the peak current amplitudes at different potentials, and fitting the data with a Boltzmann distribution of the following form: $G/G_{max} = 1/[1 + \exp\{V_{1/2} - V_t)/k\}]$, where $V_{1/2}$ is the half-activation voltage and k is the slope factor for the activation curve.

Reverse Transcription-Polymerase Chain Reaction. mRNA was isolated from fresh GC by using a Micro-Fast Track kit (Invitrogen). Reverse transcription was performed using random hexamers and enhanced avian reverse transcriptase (Sigma Chemical) under recommended conditions. PCR detection of KCNE1 was performed using gene-specific oligonucleotide primers designed to amplify a 170-bp fragment of sense: 5′-ACCCTGGGCATCATGCTGAGT-3′; antisense: 5′-TGCCGCCTGGTTTTCAATGAC-3′. Four additional primers were used for nested PCR designed to amplify an ~180-bp KCNE1 product for the outer reaction [5′-AAGCTGGAAGCACTCTAC-3′; 5′-CTCCAGAACCCGGGCCTG-3′], and an ~110-bp KCNE1 product for the inner reaction [5′-GGTTTCTTCACTCTGGGC-3′; 5′-CTTCTCCTGCCAGGCGTC-3′]. The PCR reaction mixtures (25 μl) contained 0.4 mM each 5′ and 3′ primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, and 1 unit of REDTaq DNA polymerase (Sigma). These PCR reactions were amplified for 30 cycles consisting of denaturation for 30 s at 94°C, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The PCR product was cloned into INVaF′ cells by using an Original TA Cloning kit (Invitrogen) and sequenced using T7 and M13 primers.

A similar PCR protocol was used to detect mRNA for Kv β-subunits. Primer sets were identical to those used previously to detect a 150-bp fragment of Kvβ1.1, a 141-bp fragment of Kvβ2, and a 178-bp fragment of Kvβ3 (Yuan et al., 1998).

Preparation of Granulosa Cell Lysates and Membrane Proteins. Whole-cell lysates were made from GC monolayers by standard techniques with a lysis buffer consisting of phosphate-buffered saline with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (1:500, P8340; Sigma Chemical). Lysis buffer was added to the culture dish after washing with cold phosphate-buffered saline three times. The culture dishes were scraped and the lysate was aspirated into a syringe with a 21-gauge needle to shear DNA. The lysates were rocked in the cold for 1 h and centrifuged for 10 min at 10,000g.

GC membranes were prepared by homogenizing pellets of freshly isolated GC in cold (4°C) HEPES-buffered saline (10 mM HEPES, 83 mM NaCl, and 1 mM $MgCl_2$, pH 7.9) containing protease inhibitor cocktail (1:500). A crude membrane fraction was obtained by differential centrifugation (1000g, 10 min; 100,000g, 30 min) and solubilized in cold (4°C) radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, and 1% deoxycholate; pH 7.9) containing protease inhibitor cocktail (1:500).

Protein concentrations of GC lysates and membrane protein preparations were determined by the bicinchoninic acid method (Micro BCA protein assay; Pierce Chemical, Rockford, IL). Lysates and solubilized membrane proteins were used for immunoblotting and immunoprecipitation as indicated.

Immunoblotting. Solubilized proteins were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes by the semidry transfer method. The membranes were blocked for 1 h at room temperature with 5% nonfat milk in Tris-buffered saline (TBS; 100 mM Tris, 0.9% NaCl, pH 7.5) containing 0.1% Tween 20 then incubated overnight at 4°C with primary antibody diluted in the blocking solution. Primary antibody dilutions were α-Kv1.1 (1:1000), α-Kv1.2 (1:1000), α-Kv1.3 (1:500), α-Kv1.4 (1:500), α-Kv1.5 (1:500), α-Kv1.6 (1:500), α-KvLQT1 (1:500), α-minK (1:500), and α-HERG (1:500). Unless otherwise stated, after three washes with 0.1% Tween 20/TBS, membranes were incubated for 1 h at room temperature with the appropriate HRP-conjugated secondary antibody diluted 1:1500 in 0.1% Tween 20/TBS. After four additional washes with 0.1% Tween 20/TBS, bound primary antibodies were visualized using an ECL detection system (Amersham Biosciences, Inc.) and recorded on radiographic film. Densitometric analysis was performed using Scion Image beta version 4.02 (Scion Corporation, Frederick, MD). Blots that were reprobed with antiphosphotyrosine were stripped with IgG elution buffer (Pierce Chemical) for 30 min then washed 3 × 10 min with TBS. Blots were then treated with ECL reagent as described for Western blots to confirm that antibodies were stripped completely. The blots were then reprobed with HRP-linked antiphosphotyrosine (1:500) and developed as described above.

Deglycosylation. The product of a Kv1.3 immunoprecipitation reaction was subjected to enzymatic deglycosylation by using peptide-*N*-glycosidase F (PNGase-F) according to the manufacturer's instructions (Glyko Deglycosylation Plus kit; Glyko, Inc., Novato, CA). Briefly, the glycoprotein was denatured before digestion by using SDS and β-mercaptoethanol. Samples were heated to 100°C for 5 min then cooled on ice. Excess Nonidet P-40 was added to the cooled reaction to complex the SDS then PNGase-F was added (0.2 U/ml) and the sample was incubated overnight at 37°C. A control reaction containing buffer instead of enzyme was incubated in parallel. After incubation, sample buffer was added, and the reactions were analyzed by SDS-PAGE.

Immunoprecipitation. To preclear nonspecific binding, protein G-agarose beads (Immunopure; Pierce Chemical) were incubated with samples for 2 h at 4°C with continuous gentle agitation then pelleted by centrifugation (700g). Antibodies directed against channel proteins were subsequently added to the supernatant and mixed overnight at 4°C. Immune complexes were then immobilized onto protein G-agarose beads, washed three times with cold radioimmunoprecipitation assay buffer, and eluted from the beads with SDS sample buffer containing 5% β-mercaptoethanol. Immunoprecipitated proteins and supernatant fractions were analyzed by immunoblotting as described above.

Statistical Analysis. Data are expressed as mean ± S.E.M. Significant differences between treatment groups were identified by analysis of variance with appropriate general linear models. Multiple comparisons were made using least significant difference procedure (Statistix; Analytical Software, Tallahassee, FL). Differences were considered significant when $p < 0.05$.

Results

Electrophysiological Differentiation of Delayed Rectifier K^+ Currents in Porcine Granulosa Cells. Two different outward potassium currents with distinct activation and inactivation kinetics could be elicited by a series of depolarizing test pulses from a −40-mV holding potential: a slowly activating, noninactivating current (Fig. 1) and an ultrarapidly activating, slowly inactivating current (Fig. 2). A discrete rapidly activating and inactivating (A-type) current was rarely seen when a similar voltage protocol was combined with a −80-mV holding potential (2/50 cells); this current was not further characterized.

The slowly activating, noninactivating K^+ current was present in 67% of freshly isolated GC and absent completely from GC cultured for more than 48 h. The basic electrophysiological properties and expression pattern of this current have been described previously by Mattioli et al. (1993). We refer to this current as granulosa cell I_{Ks}, because of its overt similarity to the extensively characterized I_{Ks} present in cardiac myocytes (Sanguinetti and Jurkiewicz, 1990) and other cells that coexpress KCNE1 (minK) and KCNQ1 (KvLQT1) channel proteins (Barhanin et al., 1996; Sanguinetti et al., 1996).

Granulosa cell I_{Ks} could be measured both in the presence

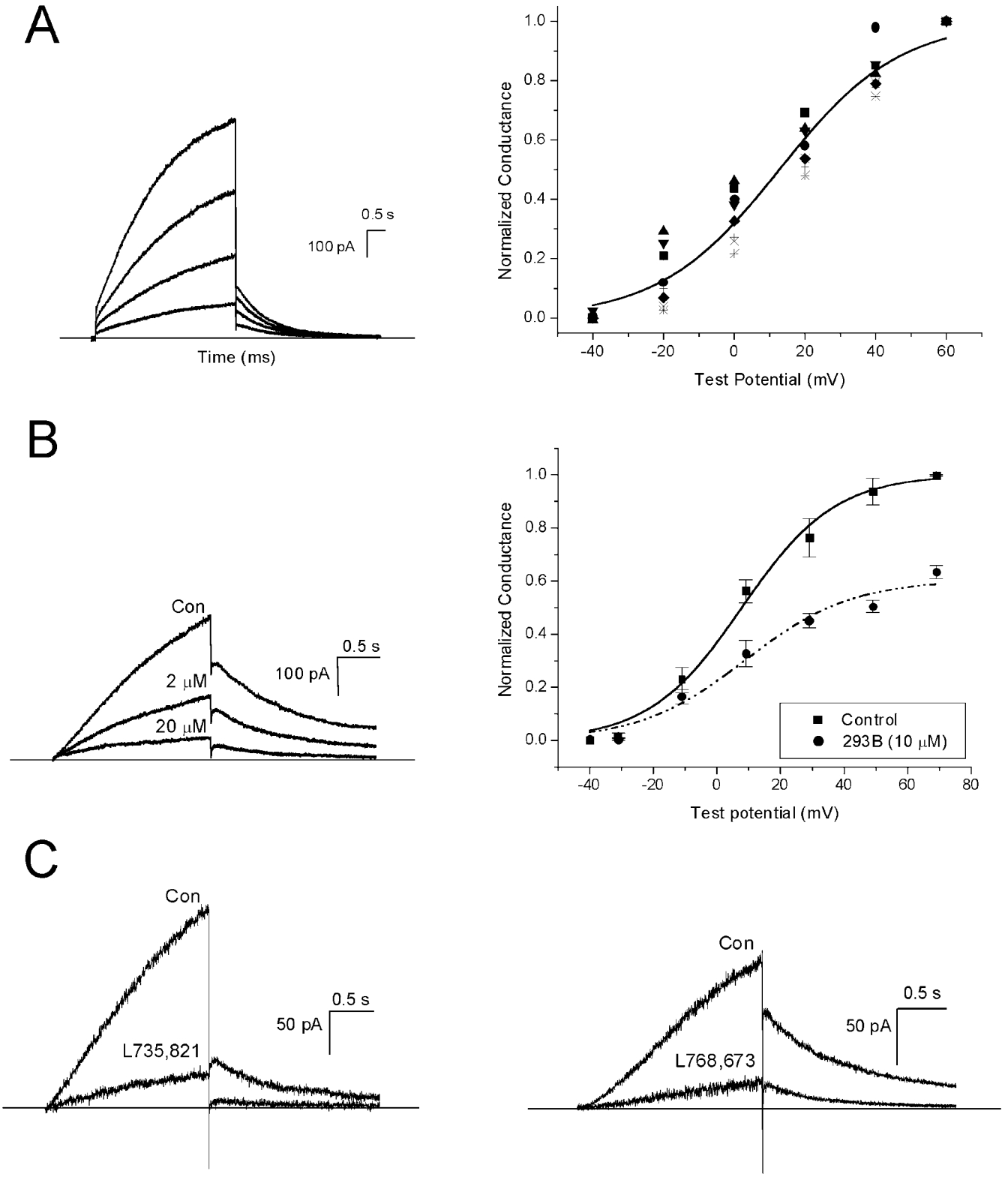


Fig. 1. A, left, slowly inactivating, nonactivating delayed rectifier K^+ current (I_{Ks}) elicited by a series of 4-s depolarizing test pulses (0 to +60 mV, step 20), followed by return to the −40-mV holding potential $[K^+]_{out}$ = 0 mM, 22°C. Right, normalized isochronal (4-s) activation curve for I_{Ks} determined from tail currents (n = 9). Continuous curve is a Boltzmann relationship fit to the mean at each test potential. The half-activation voltage ($V_{1/2}$) and slope factor (k) are 12.8 and 16.9 mV, respectively. B, left, granulosa I_{Ks} recorded at +40 mV by using the protocol described above, in the presence of increasing concentrations of the 3*R*,4*S*-enantiomer of chromanol 293B. Right, isochronal activation curves recorded in the absence and presence of 10 μM racemic 293B (n = 3). Data were normalized to the maximal control (drug-free) conductance. Continuous curves are Boltzmann relationships fit to the mean data. C, left, granulosa I_{Ks} recorded at +40 mV by using the protocol described above, before and after addition of 10 nM L-735,821. Right, granulosa I_{Ks} recorded at +40 mV by using the protocol described above, before and after addition of 20 nM L-768,673.

and absence of extracellular potassium (Fig. 1); decreasing extracellular potassium enhanced current amplitude in a manner consistent with the change in driving force (data not shown). Granulosa cell I_{Ks} activated with a half-activation voltage ($V_{1/2}$) and a slope factor (k) of 12.8 and 16.9 mV, respectively. The time course of I_{Ks} activation was described using a double exponential function, whereas that of deactivation was described by a single exponential. The fast and slow time constants of activation associated with a +60-mV test pulse were 394.7 ± 156.3 and 1370.3 ± 165.3 ms, respectively (n = 7). The deactivation time constant associated with subsequent return to −40 mV was 1028.3 ± 94.3 ms. Thus, the potassium-, time-, and voltage-dependence of GC I_{Ks}, the failure to inactivate, and the decay kinetics of the tail currents are typical of native cardiac and heterologously expressed I_{Ks} (Sanguinetti and Jurkiewicz, 1990; Barhanin et al., 1996; Sanguinetti et al., 1996). Moreover, the amplitude of GC I_{Ks} is substantially diminished by drugs described as specific I_{Ks} antagonists (Busch et al., 1996; Salata et al., 1996; Selnick et al., 1997), not only the chromanol 293B (Fig. 1B, right) and its more potent enantiomer (3*R*,4*S*)-293B (Fig. 1B, left) but also two potent benzodiazepine blockers of I_{Ks}, L735,821 (Fig. 1C, left) and L768,673 (Fig. 1C, right). Granulosa cell I_{Ks} was also inhibited by the class III antiarrhythmic drug clofilium and its *p*-nitro tertiary amine analog LY97241. The amplitude of GC I_{Ks} was reduced by >80% in the presence of these compounds at concentrations between 25 and 100 μM. For example, clofilium (50 μM) reduced I_{Ks} tail current associated with +60-mV test pulse by 91.4 ± 2.9% (n = 3). As reported previously for the cardiac slow delayed rectifier K^+ current (Arena and Kass, 1988), the LY97241-induced inhibition of GC I_{Ks} was reversible, whereas the clofilium-induced block was not. Thus, the electrophysiological and pharmacological properties of GC I_{Ks} are virtually identical to those of native and recombinant delayed rectifier currents associated with K^+ channels formed by KCNQ1 in combination with KCNE1. There was no MK-499-sensitive current in GC (n = 5).

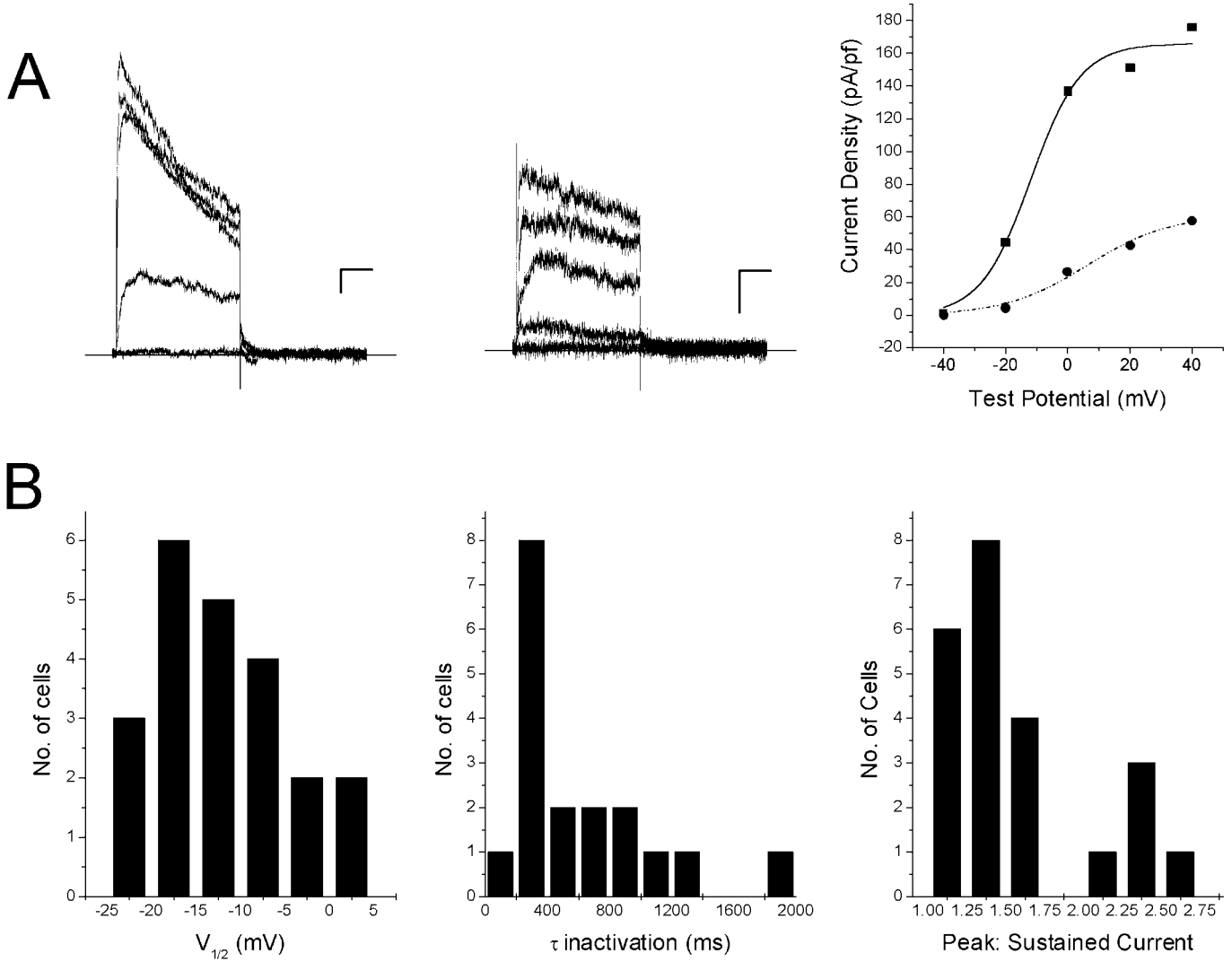


Fig. 2. A, two examples of I_{Kur} recorded from granulosa cells maintained in monolayer culture for 48 h before patch-clamp recording. The current traces in the left and center were elicited by an identical series of 1-s depolarizing test pulses (0 to +40 mV, step 20), followed by return to the −40-mV holding potential. Scale bars, 250 ms, 25 pA. Right, current density plotted as a function of test potential for the traces on the left (■, solid line) and center (●, dash-dot line). B, frequency distributions for I_{Kur} half-activation voltage (left, n = 22), inactivation time constant at +40 mV (center, n = 18), and ratio of peak to sustained current at +40 mV (n = 23).

The ultrarapidly activating, slowly inactivating delayed rectifier K^+ current (Fig. 2) will be referred to as GC I_{Kur}, because of its kinetic similarity to cardiac ultrarapid delayed rectifier currents described previously (Nattel et al., 1999). In contrast to I_{Ks}, I_{Kur} could be measured in the presence, but not the absence of extracellular potassium. Furthermore, I_{Kur} was present in both freshly isolated GC (6/16) and GC maintained in culture for up to 72 h (28/34 cells). Currents were elicited using a series of depolarizing test pulses applied from either a −40- or −80-mV potential in 30 of 34 GC exhibiting I_{Kur}, and elicited only from a −80-mV holding potential in three additional cells.

Table 1 shows the basic electrophysiological properties of I_{Kur} recorded in response to 1-s test pulses from a −40-mV holding potential, from GC that expressed I_{Kur} in the absence of I_{Ks}, after 0 to 24, 25 to 48, or 49 to 72 h of monolayer culture in serum-supplemented (10% fetal bovine serum) media. The current density, extent of time-dependent inactivation, and voltage dependence of activation of I_{Kur} varied substantially between cells on all days of culture, as illustrated by the obvious difference in the voltage dependence of activation of the current traces in Fig. 2A, and the magnitude of the standard errors in Table 1. There was no significant effect of day of culture on any parameter (Table 1). The extent of cumulative inactivation was also highly variable. For example, after 96 h of culture, there were cells with currents that showed either substantial (3/6) or no (3/6) use-dependent inactivation (Fig. 3); pulse-dependent potentiation was not seen.

The pharmacological profile of GC I_{Kur} was complex and variable. I_{Kur} amplitude was not diminished (<2% change, $n = 3$) by 293B (100 μM), L-735,821 (100 nM), or L-768,673 (100 nM). However, I_{Kur} was antagonized by three nonspecific Kv1 channel antagonists (Po et al., 1993; Grissmer et al., 1994; Yamagishi et al., 1995; Sobko et al., 1998; Rolf et al., 2000): clofilium (25–100 μM, $n = 5$), 4-AP (0.2–1 mM, $n = 4$), and verapamil (20 μM, $n = 2$) (Fig. 4C). Antagonism of GC I_{Kur} by both clofilium and verapamil was associated with an accelerated time course of decay (Fig. 4C), as described previously for these drugs' open channel block of Kv1.2 and Kv1.5 (Rampe et al., 1993; Malayev et al., 1995; Yamagishi et al., 1995). Components of whole-cell I_{Kur} were also sensitive to external TEA (Fig. 4A), as well as to the Kv1 subunit-specific toxins CTX (Fig. 4B), DTX (Fig. 4B), KTX (Fig. 4B), and MTX (data not shown). Peak I_{Kur} measured at +40 mV was diminished on average ($n = 3$): 47 ± 1% by CTX (10 nM), 25.3 ± 14% by DTX (20 nM), and 67 ± 4% by KTX (50 nM).

TABLE 1
Properties of I_{Kur} in granulosa cells cultured for 0 to 72 h

	0–24 h ($n = 4$)	25–48 h ($n = 9$)	49–72 h ($n = 10$)
Peak Current[a] (pA/pF)	23.7 ± 6.6	53.5 ± 15.4	48.1 ± 16.6
Sustained Current[a] (pA/pF)	13.3 ± 7.8	39.2 ± 11.3	36.0 ± 13.2
Ratio Peak: Sustained	1.8 ± 0.2	1.4 ± 0.1	1.6 ± 0.2
Activation			
$V_{1/2}$	−10.6 ± 4.5	−10.9 ± 2.3	−13.9 ± 2.8
k	11.0 ± 3.0	11.6 ± 1.8	9.9 ± 1.6
Inactivation (τ)	441.0 ± 134.6	656 ± 198.0	611.8 ± 169.7

Values are given as mean ± S.E.M.
[a] Peak and sustained current amplitudes were measured during and at the end 1-s test pulse to +40 mV, respectively.

Although no component of I_{Kur} in any cell tested ($n = 5$) was sensitive to 100 μM TEA, a 100-fold higher TEA concentration (10 mM) reduced peak current at +40 mV by 11 to 35%. The component of I_{Kur} that exhibited cumulative inactivation was sensitive to 10 mM TEA. Furthermore, the TEA-sensitive component of I_{Kur} activated over a more negative voltage range than the TEA-insensitive component; for example, the half-activation voltages (millivolts) determined by Boltzmann analysis of the conductance-voltage relationships for the control, TEA-insensitive, and TEA-sensitive currents shown in Fig. 4A were, respectively, −15.8, −9.4, and −20.1.

The electrophysiological and pharmacological properties of GC I_{Kur} are inconsistent with expression of any single population of Kv α-subunit homotetramers. For example, homomeric Kv1.1 channels are blocked by micromolar concentrations of TEA (Grissmer et al., 1994), whereas GC I_{Kur} is not. In contrast to the GC current, homomeric Kv1.2 channels show pulse-dependent potentiation and lack KTX sensitivity (Grissmer et al., 1994). If not coassembled with Kv1.6, Kv1.4 channels exhibit fast, N-type inactivation not typical of granulosa cell I_{Kur} (Po et al., 1993; Roeper et al., 1998). Heterologously expressed Kv1.3 currents exhibit cumulative inactivation, less sensitivity to DTX (IC_{50} = 250 nM) than either CTX (IC_{50} = 2.6 nM) and KTX (IC_{50} = 0.65 nM), and moderate TEA sensitivity; thus, Kv1.3 could account for only one component of granulosa I_{Kur} (Grissmer et al., 1994). Kv1.5 channels are not only relatively insensitive to all three toxins tested plus TEA but also lack cumulative inactivation (Grissmer et al., 1994); hence, Kv1.5 currents resemble another component of granulosa I_{Kur}. Kv1.6 channels are sensitive to DTX and TEA but insensitive to CTX and MTX (Kirsch et al., 1991; Koschak et al., 1998). On this basis, homomeric Kv1.6 channels are also unable to account completely for GC I_{Kur}.

On the basis of the data presented above, we hypothesized that GC I_{Kur} reflects heterogeneous expression of multiple Kv α-subunits, assembled as homo- and heterotetramers, sometimes associated with regulatory β-subunits. It would

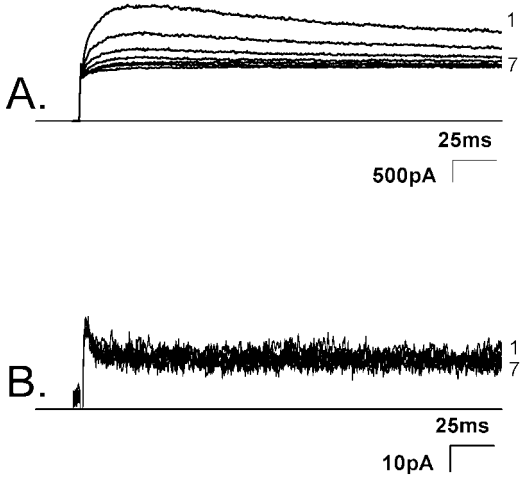


Fig. 3. Cumulative (use-dependent) inactivation of I_{Kur} was variable. Top, currents elicited from a granulosa cell maintained in monlayer culture for 96 h by a train of seven depolarizing voltage steps to +40 mV once every second from a holding potential of −80 mV. Cumulative inactivation can be visualized as a reduction in current amplitude with each successive pulse. Bottom, currents elicited from another granulosa cell maintained in monolayer culture for 96 h by using the voltage protocol described above. Cumulative inactivation is not apparent.

have been impossible to adequately test this hypothesis by measuring only electrophysiological and pharmacological characteristics of I_{Kur}, because 1) the voltage dependence, gating kinetics, and toxin sensitivity of heteromeric K^+ channels are not easily predicted and vary considerably with expression environment (Hopkins, 1998; Petersen and Nerbonne, 1999); and 2) there are no specific antagonists of Kv1.5. Accordingly, we performed additional experiments with standard molecular and biochemical techniques, as detailed below.

Potassium Channel Transcripts and Proteins Expressed in Porcine Granulosa Cells. Our goal was not only to increase knowledge about the electrophysiological and pharmacological properties of GC delayed rectifier K^+ currents but also to determine which potassium channel subunits might contribute to GC K^+ channels. To this end, we used qualitative RT-PCR and Western analysis to examine K^+ channel protein expression at the mRNA and protein levels, respectively. To address directly the issue of molecular diversity in GC Kv channel assembly, we used sequential coimmunoprecipitation and immunoblotting to document not only the presence of but also the potential for heteromultimer formation by channel subunits from the ERG (KCNH), KCNQ, KCNE, Kv1, and Kvβ families.

Immunoblots with either N- and C-terminal anti-HERG antibodies described by Pond et al. (2000), or a commercially available anti-HERG antibody (Alomone Laboratories), failed to reveal expression of ERG protein in membranes prepared from freshly isolated porcine GC, although these antibodies detected ERG1 (KCNH2) successfully in transiently transfected CHO cells, stably transfected HEK-293 cells, and guinea pig and horse heart (Fig. 5). These data are consistent with the absence in GC of an MK-499–sensitive K^+ current similar to cardiac I_{Kr}, the rapid delayed rectifier K^+ current (Sanguinetti and Jurkiewicz, 1990).

On the basis of its electrophysiological and pharmacological properties, we hypothesized that granulosa I_{Ks} was associated with channels formed by combination of the six transmembrane domain, pore-forming, α-subunit KCNQ1 with the β-subunit KCNE1. Gene-specific primers based on highly conserved regions of KCNE1-amplified RT-PCR products of the expected sizes (Fig. 6, top); sequencing confirmed the presence of transcripts encoding a protein with high homology to previously cloned KCNE1 (Fig. 6, bottom). Immunoblotting was used to confirm expression of KCNE1 protein and demonstrate expression of KCNQ1 protein; coimmunoprecipitation studies suggest strongly that these channel subunits associate in porcine GC (Fig. 7). Mattioli et al. (1993) reported, and we have confirmed, that expression of I_{Ks} by GC in monolayer culture is progressively diminished and ultimately lost over a period of 24 to 48 h. Here, we extend these findings to show that expression of the Ks channel subunits KCNQ1 and KCNE1 is diminished in GC after 24 h in primary culture (Fig. 8).

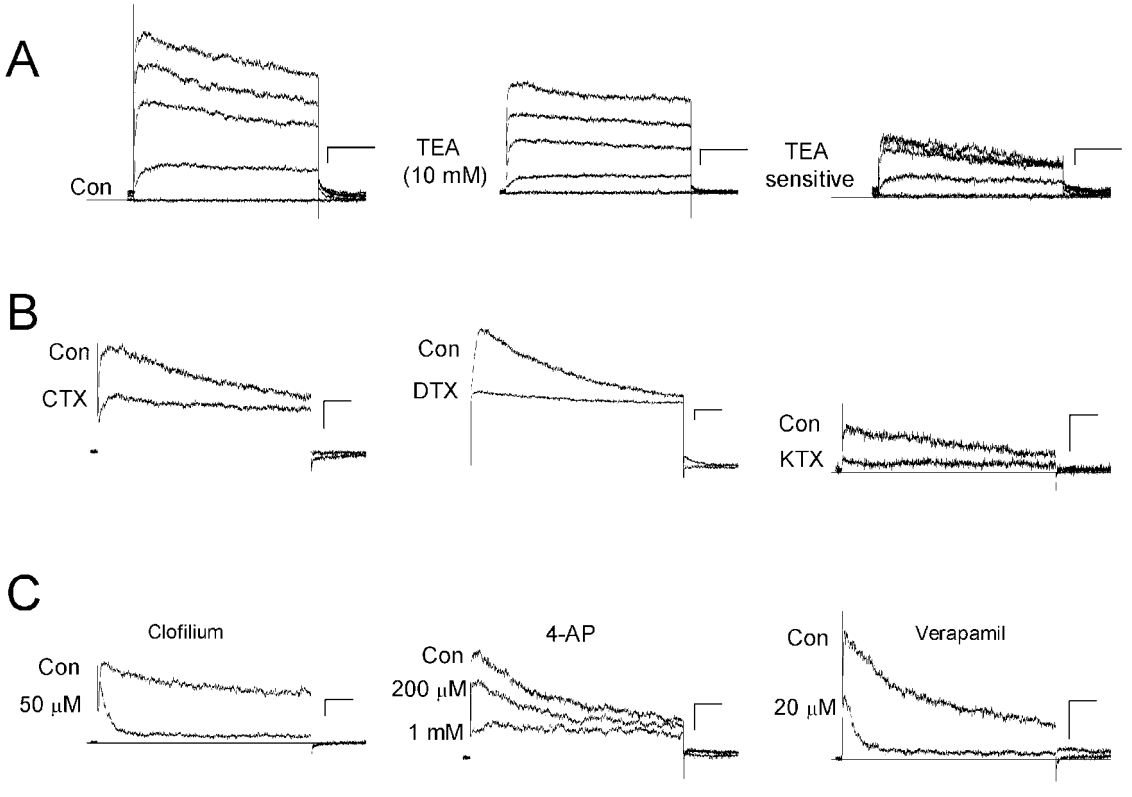


Fig. 4. A, I_{Kur} recorded from granulosa cell maintained in monolayer culture for 72 h before patch-clamp recording has a moderately TEA-sensitive component. Left, control current recorded in the absence of TEA. Middle, TEA-resistant current recorded 5 min after application of 10 mM TEA (extracellular solution with 10 mM sodium replaced by 10 mM TEA). Right, TEA-sensitive current obtained by subtracting TEA-resistant current from control. Scale bars, 250 ms, 50 pA. B, peptide toxins inhibit components of I_{Kur}. Left, inhibition by CTX (10 nM) of I_{Kur} recorded from a granulosa cell after 48 h in culture. Middle, inhibition by DTX (20 nM) of I_{Kur} recorded from a granulosa cell after 48 h in culture. Right, inhibition by KTX (50 nM) of I_{Kur} recorded from a granulosa cell after 72 h in culture. Scale bars, 125 ms, 50 pA. C, effects of nonspecific K^+ channel antagonists on I_{Kur} recorded from granulosa cells maintained in monolayer culture for 48 h before patch-clamp recording. Effects of clofilium, 4-AP, and verapamil are shown in the left, middle, and right, respectively. Similar drug effects were observed in granulosa cells cultured for 72 or 96 h. Scale bars, 125 ms, 50 pA.

We hypothesized that the variable electrophysiological and pharmacological properties of granulosa I_{Kur} reflected the presence and coassociation of multiple Kv channel subunits. This hypothesis was confirmed using the experimental approach illustrated in Figs. 8 to 11. Summary data are presented in Table 2.

As shown in Fig. 9A, antibody to Kv1.5 immunoprecipitated from CHO cells stably transfected with Kv1.5 a band of the expected molecular mass (~75 kDa, based on immunoblotting), and one additional unidentified band (~48 kDa). In contrast, the same antibody pulled down from GC not only a band spanning 68 to 80 kDa but also bands at ~60 and ~110 kDa. The molecular masses of the ^{35}S-labeled bands immunoprecipitated from GC are consistent with the reported molecular mass of Kv1 subunits. Indeed, additional immunoblotting experiments revealed that freshly isolated GC and primary cultures expressed multiple Kv1 channel proteins, including Kv1.1, Kv1.2, Kv1.3, Kv.1.4, Kv1.5, and Kv1.6 (Figs. 8 and 9).

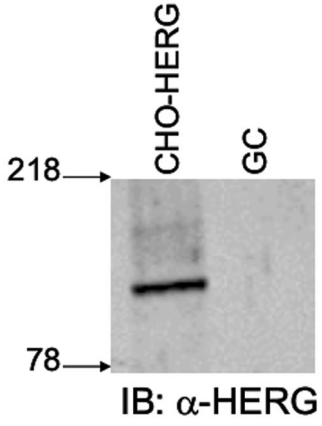
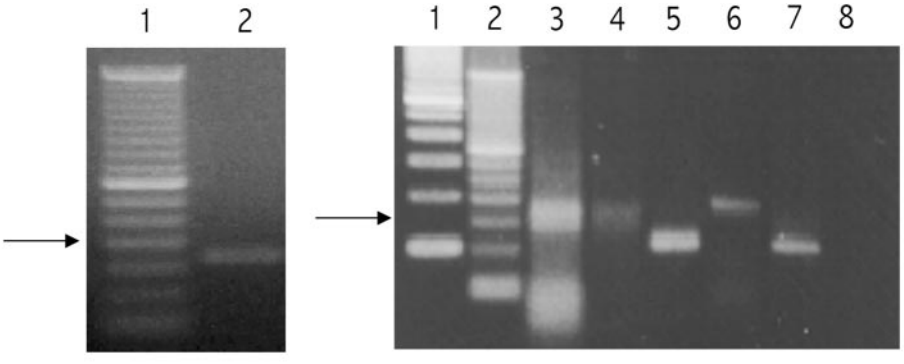


Fig. 5. ERG1 (KCNH2) is not expressed in GC. Lysates (15 μg) of CHO cells transiently transfected with HERG cDNA and porcine granulosa cells were fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted (IB) with C-terminal anti-HERG antibody. As expected, a single HERG1 protein (~145 kDa) is detected in the transfected cells (Pond et al., 2000). ERG1 is undetectable in GC. Arrows indicate molecular mass. Results representative of four experiments.

As illustrated in Fig. 9B for Kv1.5, the specificity of the Kv1 antibodies used was verified by comparing immunoblotting results obtained under standard conditions to those obtained in the presence of excess antigen (either fusion protein or synthetic peptide); only bands blocked by antigen are reported. Immunoblots of GC membranes and lysates were compared with positive and negative controls for additional verification of antibody specificity. Positive controls included lysates of Jurkat cells (positive control for Kv1.3) and CHO cells stably transfected with Kv1.5 (positive control for Kv1.5 shown in Fig. 9), as well as membranes prepared from rat brain (positive control for Kv1.1, 1.2, 1.4, and 1.6). Untransfected CHO cells and HEK-293 cells transfected with HERG served as negative controls.

On immunoblots of GC lysates and membranes, antibodies specifically recognized high- and low-molecular-mass forms of Kv1.1 (60 and 84 kDa), Kv1.2 (65 and 80 kDa), Kv1.3 (64



Pig	T	L	G	M	L	S	Y	I	R	S	K	K	L	E	H	S	H	D	P	**Y**	N	V	Y	I	**Q**	S	D
Guinea Pig	T	L	G	M	L	S	Y	I	R	S	K	K	L	E	H	S	H	D	P	**F**	N	V	Y	I	**E**	S	D
Cat	T	L	G	M	L	S	Y	I	R	S	K	K	L	E	H	S	H	D	P	**F**	N	V	Y	I	**E**	S	D
Mouse	T	L	G	M	L	S	Y	I	R	S	K	K	L	E	H	S	H	D	P	**F**	N	V	Y	I	**E**	S	D
Human	T	L	G	M	L	S	Y	I	R	S	K	K	L	E	H	S	H	D	P	**F**	N	V	Y	I	**E**	S	D

Pig	**S**	W	Q	E	K	D	K	A	**Y**	**F**	Q	A	R	V	L	E	**N**	**C**	**R**	**A**	C	**Y**	V	**I**	E	N	**Q**
Guinea Pig	**T**	W	Q	E	K	D	K	A	**F**	**F**	Q	A	R	V	L	E	**N**	**C**	**R**	**S**	C	**C**	V	**I**	E	N	**Q**
Cat	**T**	W	Q	E	K	D	K	A	**Y**	**L**	Q	A	R	V	L	E	**S**	**Y**	**K**	**A**	C	**Y**	V	**I**	E	N	**Q**
Mouse	**A**	W	Q	E	K	D	K	A	**V**	**F**	Q	A	R	V	L	E	**S**	**F**	**R**	**A**	C	**Y**	V	**I**	E	N	**Q**
Human	**A**	W	Q	E	K	D	K	A	**Y**	**V**	Q	A	R	V	L	E	**S**	**Y**	**R**	**S**	C	**Y**	V	**V**	E	N	**H**

Fig. 6. Top, KCNE1 mRNA expression in porcine granulosa cells. RT-PCR was performed using gene-specific primers. Amplified products were separated by gel electrophoresis and visualized by ethidium bromide staining. Left, predicted product of 170 bp. Arrow indicates 200 bp. Lanes: 1) 50-bp ladder; 2) porcine granulosa cell poly(A)$^+$ mRNA. Right, nested PCR with predicted products of 183 bp (outer reaction) and 110 bp (inner reaction). Arrow indicates 150 bp. Lanes: 1) 100-bp ladder; 2) 50-bp ladder; 3) porcine granulosa cell poly(A)$^+$ mRNA, outer reaction; 4) porcine granulosa cell poly(A)$^+$ mRNA, outer reaction (primers removed with spin column); 5) lane 4 product, inner reaction; 6) guinea pig heart poly(A)$^+$ mRNA, outer reaction; 7) lane 6 product, inner reaction; and lane 8, no DNA control, inner reaction. Bottom, partial deduced amino acid sequence of pig granulosa KCNE1 (GenBank accession number AF233358) compared with other KCNE clones.

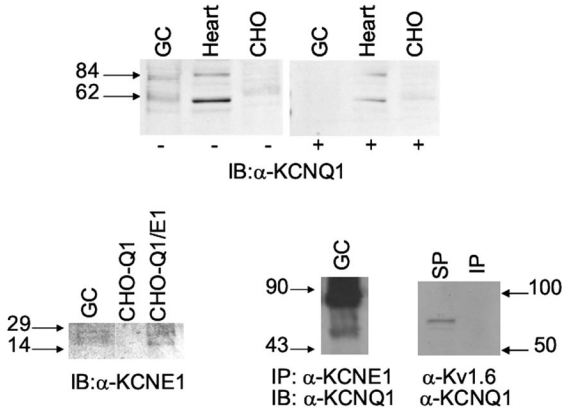


Fig. 7. KCNQ1 and KCNE1 expression in freshly isolated porcine GC. Top, KCNQ1 immunoreactivity in membranes (10 μg) prepared from GC, guinea pig heart, and untransfected CHO cells. KCNQ1 antibody (α-KCNQ1) was applied to the membrane directly (−) or after preincubation (+) with 10 μg/ml of peptide against which the antibody was generated. Bottom, left, KCNE1 immunoreactivity in membranes (20 μg) from GC, CHO cells transfected with KCNQ1 (CHO-Q1), and CHO cells cotransfected with KCNQ1 and KCNE1 (CHO-Q1/E1). In this instance, bound KCNE1 antibody (α-KCNE1) was visualized using amplified alkaline phosphatase (Bio-Rad, Hercules, CA). Three bands corresponding to differentially glycosylated forms of KCNE1 are visible. Bottom, middle, Immunoprecipitate (IP) of GC membranes by α-KCNE1, resolved by SDS-PAGE on a 4 to 20% gel, and immunoblotted (IB) with α-KCNQ1. Bottom, right, IP by α-Kv1.6 and the associated supernatant (SP) were probed with α-KCNQ1 as a negative control. Arrows indicate molecular mass.

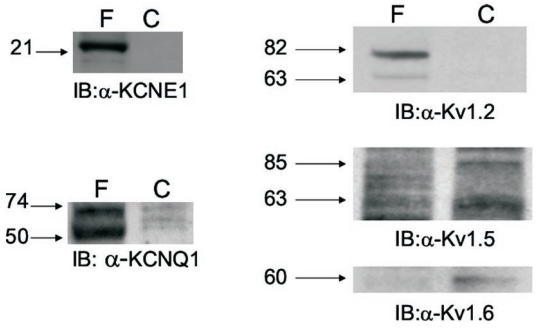


Fig. 8. Changes in expression of Kv channels during culture. Left, immunoblot (IB) of GC membrane proteins (30 μg) from fresh isolates (F) and 24 h primary cultures (C), separated by SDS-PAGE on a 4 to 20% gel, and probed with antibody to KCNE1 (top) or KCNQ1 (bottom). Right, immunoblots of membrane proteins (30 μg) isolated from freshly harvested PGC cells (F) or PGC maintained in culture (C) for 72 h. Membrane proteins were separated by SDS-PAGE on a 4 to 20% gradient gel, transferred to nitrocellulose, and detected with anti-Kv1.2 (top), anti-Kv1.5 (middle), or anti-Kv1.6 (bottom). Arrows indicate molecular mass.

and 88 kDa), Kv1.4 (88 and 110 kDa), Kv1.5 (62 and 85 kDa), and Kv1.6 (60 and 88 kDa). The lower molecular mass for Kv1.6 (60 kDa; Fig. 8) has been described in other cell types (Scott et al., 1994; Koch et al., 1997). An 88-kDa form of Kv1.6 has also been described previously, although the basis for the higher molecular mass is not known (Attali et al., 1997). Addition of N-linked oligosaccharide is unlikely because Kv1.6 lacks the *N*-glycosylation site present in Kv1.1, Kv1.2, Kv1.3, Kv1.4, and Kv1.5 (Shi and Trimmer, 1999). The low- and high-molecular-mass forms of Kv1.1, Kv1.2, and Kv1.4 have been shown previously to correspond, respectively, to incompletely glycosylated (high mannose), immature, and fully glycosylated (sialylated) mature forms of these channel subunits (Scott et al., 1994; Shi and Trimmer, 1999; Manganas and Trimmer, 2000). High- and low-molecular-mass forms of Kv1.3 and Kv1.5 have also be reported and are suggested to reflect post-translational modification of these proteins, which contain multiple phosphorylation sites in addition to the conserved *N*-glycosylation site (Attali et al., 1997; Bowlby et al., 1997; Chung and Schlichter, 1997; Sobko et al., 1998; Yuan et al., 1998). We confirmed the PNGase-F sensitivity of the high molecular form of Kv1.3 expressed in cultured but not freshly isolated GC, to provide a direct correlation between the high-molecular-mass form of this α-subunit and the presence of mature N-linked oligosaccharide. PNGase-F digestion shifted the mobility of Kv1.3 from 88 to 64 kDa (data not shown).

We used coimmunoprecipitation (pull-down) to determine which, if any, of the Kv1 α-subunits contributed to heterotetramer formation in porcine GC. Table 2 summarizes the results of a series of experiments similar to those shown in Fig. 9. It should be noted that the scoring system used in Table 2 to depict the apparent relative abundance of the different molecular mass forms of the various Kv channel subunits reflects not only their actual expression but also differences between antibody efficiency and interexperimental variation in the chemiluminescence reactions.

To define the nature of some of the oligomeric assemblies of Kv α-subunits in porcine GC, we used a strategy similar to that of Shamotienko et al. (1997). GC membranes were immunoprecipitated with antibody against Kv1.1, 1.2, or 1.5 then the resultant precipitate and supernatant fractions were immunoblotted with the same antibody to demonstrate complete precipitation of the targeted Kv channel protein from the membrane fraction. These supernatants were then subjected to additional rounds of immunoprecipitation and immunoblotting with the other Kv antibodies to determine

TABLE 2

Molecular basis for I_{Kur}

Interactions between different Kv α-subunits as demonstrated by co-immunoprecipitation. Where low- and high-molecular-mass forms of Kv protein were detected, the relative amounts that coassociate are indicated. Minus sign signifies undetectable by immunoblotting. Plus sign indicates apparent relative abundance (+ < ++ < +++). Each cell represents data obtained by immunoprecipitation (IP) and immunoblotting (IB) of multiple membrane preparations from fresh granulosa cells (n = 2–5). Typically, membranes were prepared using granulosa cells harvested from the small and medium follicles on 150 to 300 pig ovaries.

IP Antibody	IB Antibody											
	1.1		1.2		1.3		1.4		1.5		1.6	
	60kD	84kD	65kD	80kD	64kD	88kD	88kD	110kD	62kD	85kD	60kD	88kD
1.1			+	++	+++		++	+	++	+	−	−
1.2	++	+			++		++	+++	++	++	+	−
1.3	+++	+	−	−			+	−		+++	−	−
1.4	+	−	−	++	−						−	++
1.5	+	++	+	++	+		++	++			++	+
1.6	+				−		++	+++	+	+		

which (if any) of the possible hetero-oligomeric subunit combinations were present. These experiments indicated that Kv1.1 is not found in complexes without Kv1.5 but can be found without Kv1.2. In contrast, Kv1.5 is found in complexes that have neither Kv1.1 nor Kv1.2.

To detect temporal differences in the expression of the various Kv α-subunits, immunoblots performed on membranes prepared from freshly isolated GC were compared with immunoblots performed using membranes prepared from 72-h monolayer cultures. Expression of Kv1.1, Kv1.2 (Fig. 8), and Kv1.4 (data not shown) was greater in freshly isolated GC than 72-h primary cultures. The opposite was true for Kv1.3, Kv1.5, and Kv1.6 (Figs. 8 and 10). Kv1.3 was undetectable in two of six membrane preparations from freshly isolated granulosa cells. The PNGase-F-sensitive form of Kv1.3 (80 kDa) was found only in cultured GC (Fig. 10). Similarly, high-molecular-mass forms of Kv1.5 were expressed to a greater extent in cultured than fresh GC (Fig. 8). Interestingly, Kv1.3 was subject to constitutive tyrosine phosphorylation in freshly isolated and cultured GC; anti-phosphotyrosine consistently detected the same bands as anti-Kv1.3, on Western blots (Fig. 10) and immunoprecipitates (data not shown).

Heteromultimeric assembly of Kvα and Kvβ assembly is known to contribute to K^+ current diversity in cardiac, neuronal, and other cells. To determine whether Kv β-subunits are expressed and capable of association with Kv α-subunits in GC, we used PCR to look for mRNA encoding Kvβ1, Kvβ2, and Kvβ3 and found that message for Kvβ2 is expressed in GC (Fig. 11). In addition, we used Western analysis to detect Kvβ1.3 in GC membrane fractions immunoprecipitated with antibody against Kvβ1.3 and three Kv α-subunits. As shown in Fig. 11, Kvβ1.3 is expressed in freshly isolated GC and coassociates with Kv1.2 and Kv1.5, but not Kv1.1. It is unclear why immunoblotting successfully detected Kvβ1.3 protein, when PCR with primers complementary to a C-terminal sequence shared by all Kvβ1.x subunits did not yield any product (Fig. 11). Product of the expected size was not seen using PCR conditions identical to those published for pulmonary artery smooth muscle (Yuan et al., 1998), or under conditions where buffer (Opti-Prime PCR Optimization kit; Stratgene, La Jolla, CA) and annealing temperature (40–56°C gradient, Tgradient Thermocycler; Whatman Biometra GmbH, Niedersachsen, Germany) were varied. Optimization of PCR conditions for Kvβ1.x was not pursued after Kvβ1.3 protein was demonstrated by immunoblotting.

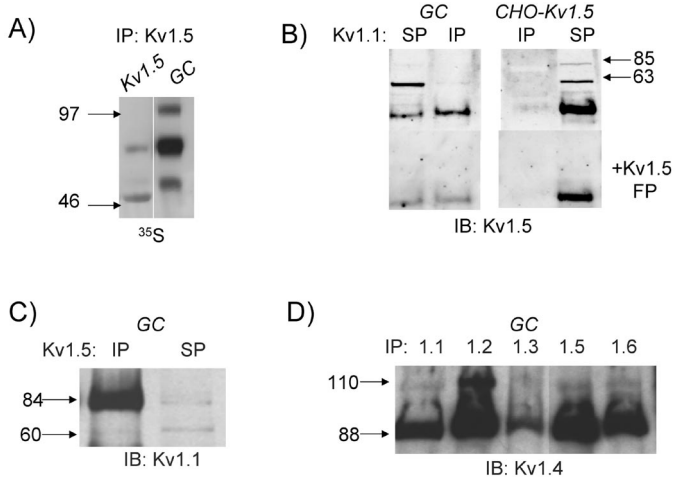


Fig. 9. Heteromultimeric coassembly of Kv channel subunits in porcine granulosa cells. Immunoprecipitation (IP) from the membrane protein fraction was performed then the resulting IP and supernatant (SP) were resolved by SDS-PAGE (4 to 20% gradient gels) and analyzed by immunoblotting (IB). A, IP of ^{35}S-labeled proteins from Chinese hamster ovary cells stably transfected with Kv1.5 (Kv1.5) and porcine GC. B, proteins from GC and Chinese hamster ovary cells stably transfected with Kv1.5 (CHO-Kv1.5) were IP with antibody to Kv1.1. The IP and SP were then IB with anti-Kv1.5, in the absence and presence (bottom) of Kv1.5 fusion protein (FP). C, membrane proteins from porcine granulosa cells were IP with antibody to Kv1.5. The IP and SP were probed with antibody to Kv1.1. D, proteins IP with antibodies (rabbit polyclonal) to Kv1.1-Kv1.6 were IB with antibody (mouse monoclonal) to Kv1.4. Arrows indicate molecular mass.

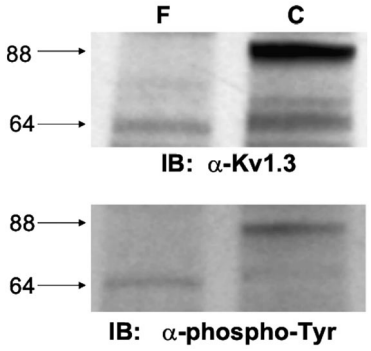


Fig. 10. Expression and phosphorylation of Kv1.3 in fresh and cultured porcine granulosa cells. Western blot of membrane proteins isolated from freshly harvested PGC cells (F) or PGC maintained in culture for 72 h (C). Membrane proteins were separated by SDS-PAGE on a 4 to 20% gradient gel, transferred to nitrocellulose, and detected with anti-Kv1.3 (top). The blot was then stripped and reprobed with HRP-linked anti-phosphotyrosine (bottom).

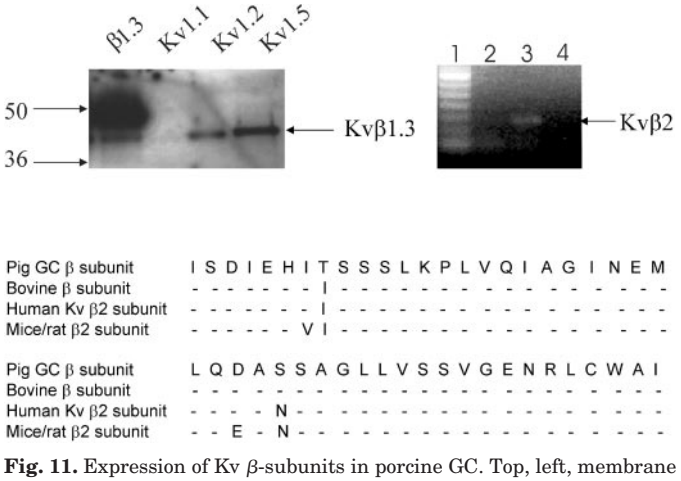


Fig. 11. Expression of Kv β-subunits in porcine GC. Top, left, membrane proteins from porcine GC were immunoprecipitated with antibody to Kvβ1.3, Kv1.1, Kv1.2, or Kv1.5 and resolved by SDS-PAGE (4 to 20% gradient gels). The Western blot was probed with antibody to Kvβ1.3. The wide band at 50 kDa in the lane sequentially immunoprecipitated then immunoblotted with rabbit anti-Kvβ1.3 is IgG detected by the anti-rabbit secondary antibody. This band is lacking in the other lanes, because Kv α-subunits were immunoprecipitated with mouse monoclonal antibodies. Left arrows indicate molecular mass standards. Right arrow (~40 kDa) indicates Kvβ1.3 protein. Top, right, mRNA for Kvβ2, but not Kvβ1 nor Kvβ3, is detected in porcine GC. RT-PCR was performed using gene-specific primers and poly(A)$^+$ mRNA. Amplified products were separated by gel electrophoresis and visualized by ethidium bromide staining. Lanes: 1) 50-bp ladder; 2) primers for β1, 3) primers for β2, and 4) primers for β3. Right arrow (~140 bp) indicates Kvβ2 product. Bottom, partial deduced amino acid sequence of pig granulosa Kvβ2 (GenBank accession number AF348084), compared with previous clones. Dashes indicate homology to Pig GC Kvβ2.

Discussion

Kv channels not only influence the electrical properties of excitable and nonexcitable cells but also regulate cell proliferation and differentiation (Attali et al., 1997; Sobko et al., 1998; Kotecha and Schlichter, 1999; Rane, 1999). K^+-selective channel pores are formed by tetrameric complexes of integral membrane proteins with six transmembrane-spanning domains. Formation of homo- and heterotetramers of Kv α-subunits has been demonstrated not only in heterologous expression systems but also native cells (Scott et al., 1994; Attali et al., 1997; Koch et al., 1997; Shamotienko et al., 1997; Sobko et al., 1998; Yuan et al., 1998; Schmidt et al., 1999). The subunit composition of Kv channels influences the expression, and electrophysiological and pharmacological properties of the associated currents (Hopkins, 1998). Kv channel expression and function may be modulated not only by coassociation of the various pore-forming α-subunits with accessory (β)-subunits and other regulatory proteins but also by post-translational modifications of either the Kv α- or Kv β-subunits (Martens et al., 1999; Petersen and Nerbonne, 1999). Several K^+ channel subunits are typically expressed in a single cell. The extensive diversity in Kv channels has made it difficult to determine definitively the molecular identify of native K^+ currents in many cases (Attali et al., 1997; Shamotienko et al., 1997; Sobko et al., 1998; Yuan et al., 1998; Schmidt et al., 1999).

A variety of K^+ currents with different activation and inactivation kinetics have been described in porcine GC subjected to whole-cell patch clamp. In GC cultured for 2 days in serum-free media containing FSH (10 ng/ml) then an additional 3 to 5 days in FSH-free media, Kusaka et al. (1993) described a rapidly inactivating transient outward (A-type) current activated from a −70-mV holding potential, and a rapidly activating noninactivating delayed rectifier K^+ current activated from a holding potential of −30 mV. Both currents were sensitive to 4-AP but only the delayed rectifier was sensitive to TEA (10 mM). In freshly isolated GC and GC maintained cultured for up to 3 days in serum-containing media (10% fetal bovine serum), Mattioli et al. (1991, 1993) described two currents: a slowly activating, noninactivating, TEA- and 4-AP-insensitive K^+ current that disappeared after 24 h in monolayer culture, and a rapidly activating K^+ current with an inactivation time constant that increased from 10 to 300 ms over 72 h in culture. The latter could be activated from a holding potential of −70 but not −40 mV and was sensitive to 4-AP. We recorded routinely a slowly activating, noninactivating delayed rectifier K^+ current and an ultrarapidly activating, slowly inactivating K^+ current. A rapidly inactivating A-type current was elicited from a −80-mV holding potential in less than 5% of screened GC.

The slowly activating, noninactivating current in Fig. 1 is identical to that described previously (Mattioli et al., 1991, 1993). Its electrophysiological properties and drug sensitivity are consistent with those of I_{Ks} currents and channels formed by coassociation of KCNQ1 and KCNE1 proteins (Barhanin et al., 1996; Busch et al., 1996; Sanguinetti et al., 1996; Selnick et al., 1997). In fact, both of these channel proteins are expressed in GC. The disappearance of I_{Ks} as GC differentiate in culture, along with the observation that the current is inhibited by luteinizing hormone, cAMP, and protein kinase C, has led to the speculation that I_{Ks} may play a specific role in granulosa cell maturation (Mattioli et al., 1991, 1993; L. C. Freeman, unpublished observations). Our identification not only of KCNQ1 and KCNE1 as the molecular correlates of GC I_{Ks} but also of 293B, L-735,821, and L-768,673 as specific antagonists of the current, will facilitate further investigation of its functional role. Additional experiments will be required to determine the basis for tissue-specific differences in I_{Ks} modulation, particularly to address the discrepant effects of cAMP on GC I_{KS} compared with recombinant KCNQ1/KCNE1 currents (Blumenthal and Kaczmarek, 1992) and native cardiac currents in guinea pig (Walsh et al., 1989) and pig (L. C. Freeman, unpublished observations).

The ultrarapidly activating, slowly inactivating K^+ current recorded from GC by using whole-cell patch clamp seems to reflect overlapping components carried by homomeric and heteromeric Kv channels. The variability in the electrophysiological and pharmacological properties of granulosa cell delayed rectifier currents described here and elsewhere (Kusaka et al., 1993; Mattioli et al., 1993) is not surprising given the results shown in Figs. 7 to 10. GC can express at least six Kv α-subunits, and their expression varies with time in culture. The diversity of granulosa cell K^+ currents is further increased by the potential for not only coassembly of nonidentical Kv α-subunits but also modulation by accessory subunits. Our data indicate that the potential for K^+ channel diversity in GC is comparable with that seen in the central nervous system. Further experiments are required to determine whether GC also expresses non–Shaker-related Kv channel proteins (Kv2.x, Kv3.x, Kv4.x, etc.).

Heterotetrameric complexes of α-subunits have been demonstrated previously among the following: Kv1.1/Kv1.2, Kv1.2/Kv1.4, Kv1.2/Kv1.3, Kv1.4/Kv1.6, Kv1.5/Kv1.2, Kv1.5/Kv1.4, Kv1.1/Kv1.2/Kv1.4, Kv1.1/Kv1.2/Kv1.6, Kv1.1/Kv1.2/Kv1.3, Kv1.2/Kv1.3/Kv1.4, and Kv1.2/Kv1.4/Kv1.6 (Koch et al., 1997; Shamotienko et al., 1997; Koschak et al., 1998; Sobko et al., 1998). In addition, the potential for heteromultimer formation has been demonstrated in oligodendrocyte progenitors for Kv1.4, Kv1.5, and Kv1.6 (Attali et al., 1997; Schmidt et al., 1999). In GC that express Kv1.1 to Kv1.6 proteins, we were able to demonstrate many of these Kv α-subunit coassociations (Table 2).

The strong coassociation observed between the glycosylated forms of Kv1.4 and Kv1.6 is noteworthy and may explain our failure to detect rapidly inactivating A-type currents in a significant number of GC. Heteromeric expression of Kv1.4 and Kv1.6 has been shown to result in a slowly inactivating current (Roeper et al., 1998), because the N terminus of Kv1.6 possesses an N-type inactivation prevention domain, which prevents the fast, N-type inactivation typically associated with homomeric Kv1.4 channels. A potentially significant pattern of coassociation was also evident between the 60-kDa form of Kv1.1 and the low-molecular-mass forms of Kv1.2, Kv1.3, Kv1.4, and Kv1.5. These relationships may reflect a physiological role for Kv1.1 in limiting surface expression of the other Kv subunits. Kv1.1 has been reported to have a dominant negative effect on the surface expression of Kv1.2 and Kv1.4 (Manganas and Trimmer, 2000).

Kv β-subunits can also affect dramatically both the gating and cell surface expression of coassociated Kv α-subunits (Martens et al., 1999). Our data demonstrate that GC ex-

press at least two β-subunits encoded by distinct genes. Kvβ1 and Kvβ2 family members not only interact with α-subunits via distinct functional stoichiometries ($\alpha_4\beta_n$ for Kvβ1 and $\alpha_4\beta_4$ for Kvβ2) but also affect Kv currents in distinct manners (Xu et al., 1998; Martens et al., 1999). For example, Kvβ1.3 converts Kv1.5 from a delayed rectifier channel to one with rapid, but partial inactivation, whereas Kvβ2.1 shifts the state-state activation and inactivation of Kv1.5 without inducing rapid inactivation. Kvβ2 subunits have also been shown to enhance *N*-glycosylation and/or surface expression of associated Kv α-subunits, including Kv1.1, Kv1.2, Kv1.3, and Kv1.6. Clearly, the presence of Kvβ1 and Kvβ2 accessory subunits in GC could contribute substantially to Kv current diversity, not only by influencing the electrophysiological properties of expressed delayed rectifier currents but also by controlling the number of α-subunits available for tetramer assembly.

Phosphorylation has been shown also to modulate current amplitudes and kinetics by influencing interactions between not only α- and β-subunits but also Kv channel complexes and other intracellular proteins (Bowlby et al., 1997; Martens et al., 1999; Rane, 1999). Basal tyrosine phosphorylation of Kv1.3 suggests that the activity of GC Kv channels may be influenced by signaling pathways associated with intraovarian growth factors (Steele and Leung, 1993). Tyrosine phosphorylation has been identified previously as an important influence on the activity of Kv1.3 (Bowlby et al., 1997; Rane, 1999), an ion channel with a well established role in modulating proliferation and differentiation of other nonexcitable cells (Kotecha and Schlichter, 1999; Rane, 1999).

As GC spontaneously luteinized in culture, expression of Kv1.3 increased. The mature, fully glycosylated form of Kv1.3 was present only in cultured GC. Whole-cell K^+ currents recorded from cultured GC contained a TEA-sensitive component that exhibited cumulative inactivation, consistent with expression of Kv1.3 homotetramers. It will be interesting to determine what role, if any, Kv1.3 plays in granulosa cell maturation. Increased expression of Kv1.3 has been positively correlated with days in culture and acquisition of a proliferative phenotype in microglia (Kotecha and Schlichter, 1999).

Associating specific K^+ channels with cellular processes in GC will be technically challenging as a result of the molecular diversity. However, it will be critical to define the roles of specific K^+ channels in granulosa cell proliferation, differentiation, and apoptosis, and the temporal pattern of K^+ channel expression during the estrous cycle, because these proteins may represent either novel targets for assisted reproduction or potential sites of toxicity for drugs designed to act on channels in other tissues, including heart, brain, and lymphocytes. We document here distinct differences in the pharmacological sensitivities and temporal expression patterns of voltage-gated K^+ currents and channel proteins in fresh isolates and primary cultures of porcine GC. Our findings provide an essential background for experimental definition of granulosa K^+ channel function(s).

Acknowledgments

We thank Jeremy J. Lippold for help obtaining some of the patch-clamp data. Suhasni Ganta provided expert technical assistance with cell culture and protein chemistry.

References

Arena JP and Kass RS (1988) Block of heart potassium channels by clofilium and its tertiary analogs: relationship between drug structure and type of channel blocked. *Mol Pharmacol* **34:**60–66.
Attali B, Wang N, Kolot A, Sobko A, Cherepanov V, and Soliven B (1997) Characterization of delayed rectifier Kv channels in oligodendrocytes and progenitor cells. *J Neurosci* **17:**8234–8245.
Barano JL and Hammond JM (1985) Serum-free medium enhances growth and differentiation of cultured pig granulosa cells. *Endocrinology* **116:**51–58.
Barhanin J, Lesage F, Guillemare E, Fink M, Lazdunski M, and Romey G (1996) K(V)LQT1 and IsK (minK) proteins associate to form the I(Ks) cardiac potassium current. *Nature (Lond)* **384:**78–80.
Blumenthal EM and Kaczmarek LK (1992) Modulation by cAMP of a slowly activating potassium channel expressed by *Xenopus* oocytes. *J Neurosci* **12:**290–296.
Bowlby MR, Fadool DA, Holmes TC, and Levitan IB (1997) Modulation of the Kv1.3 potassium channel by receptor tyrosine kinases. *J Gen Physiol* **110:**601–610.
Busch AE, Suessbrich H, Waldegger S, Sailer E, Greger R, Lang H, Lang F, Gibson KJ, and Maylie JG (1996) Inhibition of IKs in guinea pig cardiac myocytes and guinea pig IsK channels by the chromanol 293B. *Pflueg Arch Eur J Physiol* **432:**1094–1096.
Chung I and Schlichter LC (1997) Native Kv1.3 channels are upregulated by protein kinase C. *J Membr Biol* **156:**73–85.
Grissmer S, Nguyen AN, Aiyar J, Hanson DC, Mather RJ, Gutman GA, Karmilowicz MJ, Auperin DD, and Chandy KG (1994) Pharmacological characterization of five cloned voltage-gated K^+ channels, types Kv1.1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. *Mol Pharmacol* **45:**1227–1234.
Hopkins WF (1998) Toxin and subunit specificity of blocking affinity of three peptide toxins for heteromultimeric, voltage-gated potassium channels expressed in *Xenopus* oocytes. *J Pharmacol Exp Ther* **285:**1051–1060.
Kirsch GE, Drewe JA, Verma S, Brown AM, and Joho RH (1991) Electrophysiological characterization of a new member of the RCK family of rat brain K^+ channels. *FEBS Lett* **278:**55–60.
Koch RO, Wanner SG, Koschak A, Hanner M, Schwarzer C, Kaczorowski GJ, Slaughter RS, Garcia ML, and Knaus HG (1997) Complex subunit assembly of neuronal voltage-gated K^+ channels. Basis for high-affinity toxin interactions and pharmacology. *J Biol Chem* **272:**27577–27581.
Koschak A, Bugianesi RM, Mitterdorfer J, Kaczorowski GJ, Garcia ML, and Knaus HG (1998) Subunit composition of brain voltage-gated potassium channels determined by hongotoxin-1, a novel peptide derived from *Centruroides Limbatus* venom. *J Biol Chem* **273:**2639–2644.
Kotecha SA and Schlichter LC (1999) A Kv1.5 to Kv1.3 switch in endogenous hippocampal microglia and a role in proliferation. *J Neurosci* **19:**10680–10693.
Kusaka M, Tohse N, Nakaya H, Tanaka T, Kanno M and Fujimoto S (1993) Membrane currents of porcine granulosa cells in primary culture: characterization and effects of luteinizing hormone. *Biol Reprod* **49:**95–103.
Malayev AA, Nelson DJ, and Philipson LH (1995) Mechanism of clofilium block of the human Kv1.5 delayed rectifier potassium channel. *Mol Pharmacol* **47:**198–205.
Manganas LN and Trimmer JS (2000) Subunit composition determines Kv1 potassium channel surface expression. *J Biol Chem* **275:**29685–29693.
Martens JR, Kwak Y, and Tamkun MM (1999) Modulation of Kv Channel α/β subunit interactions. *Trends Cardiovasc Med* **9:**253–258.
Mattioli M, Barboni B, Bacci ML, and Seren E (1990) Maturation of pig oocytes: observations on membrane potential. *Biol Reprod* **43:**318–322.
Mattioli M, Barboni B, and DeFelice LJ (1993) Calcium and potassium currents in porcine granulosa cells maintained in follicular or monolayer tissue culture. *J Membr Biol* **134:**75–83.
Mattioli M, Barboni B, and Seren E (1991) Luteinizing hormone inhibits potassium outward currents in swine granulosa cells by intracellular calcium mobilization. *Endocrinology* **129:**2740–2745.
Mealing G, Morley P, Whitfield JF, Tsang BK, and Schwartz JL (1994) Granulosa cells have calcium-dependent action potentials and a calcium-dependent chloride conductance. *Pflueg Arch Eur J Physiol* **428:**307–314.
Nattel S, Yue L, and Wang Z (1999) Cardiac ultrarapid delayed rectifiers: a novel potassium current family of functional similarity and molecular diversity. *Cell Physiol Biochem* **9:**217–226.
Petersen KR and Nerbonne JM (1999) Expression environment determines K^+ current properties: Kv1 and Kv4 α-subunit-induced K^+ Currents in mammalian cell lines and cardiac myocytes. *Pflueg Arch Eur J Physiol* **437:**381–392.
Po S, Roberds S, Snyders DJ, Tamkun MM, and Bennett PB (1993) Heteromultimeric assembly of human potassium channels. Molecular basis of a transient outward current? *Circ Res* **72:**1326–1336.
Pond AL, Scheve BK, Benedict AT, Petrecca K, Van Wagoner DR, Shrier A, and Nerbonne JM (2000) Expression of distinct ERG proteins in rat, mouse, and human heart. Relation to functional I(Kr) channels. *J Biol Chem* **275:**5997–6006.
Rampe D, Wible B, Fedida D, Dage RC, and Brown AM (1993) Verapamil blocks a rapidly activating delayed rectifier K+ channel cloned from human heart. *Mol Pharmacol* **44:**642–648.
Rane SG (1999) Ion channels as physiological effectors for growth factor receptor and Ras/ERK signaling pathways. *Adv Second Messenger Phosphoprot Res* **33:**107–127.
Roeper J, Sewing S, Zhang Y, Sommer T, Wanner SG, and Pongs O (1998) NIP domain prevents N-Type inactivation in voltage-gated potassium channels. *Nature (Lond)* **391:**390–393.
Rolf S, Haverkamp W, Borggrefe M, MuBhoff, Echkardt L., Mergenthaler J, Snyders DJ, Pongs O, Speckman, E-J., Breithardt G, et al. (2000) Effects of antiarrhythmic drugs on cloned cardiac voltage-gated potassium channels expressed in *Xenopus* oocytes. *Naunyn-Schmiederberg's Arch Pharmacol* **362:**22–31.
Salata JJ, Jurkiewicz NK, Sanguinetti MC, Siegl PK, Claremon DA, Remy DC, Elliot JM, and Libby BE (1996) The novel class III anti-arrhythmic agent, L-735,821 is

a potent and selective blocker of IKs in guinea pig ventricular myocytes (Abstract). *Circulation* **94:**I-529.
Salustri A, Hascall V, Camaioni A, and Wyanagishita M (1993) Oocyte-granulosa cell interactions, in *The Ovary* (Adashi EY and Leung CK eds) pp 209–225, Raven Press, New York.
Sanguinetti MC, Curran ME, Zou A, Shen J, Spector PS, Atkinson DL, and Keating MT (1996) Coassembly of K(V)LQT1 and minK (IsK) proteins to form cardiac I(Ks) potassium channel. *Nature (Lond)* **384:**80–83.
Sanguinetti MC and Jurkiewicz NK (1990) Two components of cardiac delayed rectifier K^+ current: differential sensitivity to block by class III antiarrhythmic agents. *J Gen Physiol* **96:**195–215.
Schmidt K, Eulitz D, Veh RW, Kettenmann H, and Kirchhoff F (1999) Heterogeneous expression of voltage-gated potassium channels of the *Shaker* family (Kv1) in oligodendrocyte progenitors. *Brain Res* **843:**145–160.
Scott VES, Muniz ZM, Sewing S, Lichtinghagen R, Parcej DN, Pongs O, and Dolly JO (1994) Antibodies specific for distinct Kv subunits unveil a heterooligomeric basis for subtypes of α-dendrotoxin-sensitive K^+ channels in bovine brain. *Biochemistry* **33:**11084–11088.
Selnick HG, Liverton NJ, Baldwin JJ, Butcher JW, Claremon DA, Elliott JM, Freidinger RM, King SA, Libby BE, McIntyre CJ, et al. (1997) Class III antiarrhythmic activity *in vivo* by selective blockade of the slowly activating cardiac delayed rectifier potassium current I_{Ks} by (*R*)-2-(2,4-trifluoromethyl)-*N*-[2-oxo-5-phenyl-1-(2,2,2-trifluoroethyl)-2,3-dihydro-1*H*-benzo[e][1,4]diazepin-3-Yl]acetamide. *J Med Chem* **40:**3865–3868.
Shamotienko OG, Parcej DN, and Dolly JO (1997) Subunit combinations defined for K^+ channel Kv1 subtypes in synaptic membranes from bovine brain. *Biochemistry* **36:**8195–8201.
Shi G and Trimmer JS (1999) Differential asparagine-linked glycosylation of voltage-gated K^+ channels in mammalian brain and in transfected Cells. *J Membr Biol* **168:**265–273.
Sobko A, Peretz A, Shirihai O, Etkin S, Cherepanova V, Dagan D, and Attali B (1998) Heteromultimeric delayed-rectifier K^+ channels in Schwann cells: developmental expression and role in cell proliferation. *J Neurosci* **18:**10398–10408.
Steele GL and Leung P (1993) Signal transduction mechanisms in ovarian cells, in *The Ovary* (Adashi EY and Leung CK eds) pp 113–128, Raven Press, New York.
Walsh KB, Begenisich T, and Kass RS (1989) β-Adrenergic modulation of cardiac ion channels: differential temperature sensitivity of potassium and calcium currents. *J Gen Physiol* **93:**841–854.
Xu J, Yu W, Wright JM, Raab RW, and Li M (1998) Distinct functional stoichiometry of potassium channel β subunits. *Proc Natl Acad Sci USA* **95:**1846–1851.
Yamagishi T, Ishii K, and Taira N (1995) Antiarrhythmic and bradycardic drugs inhibit currents of cloned K^+ channels, Kv1.2 and Kv1.4. *Eur J Pharmacol* **281:** 151–159.
Yuan XJ, Wang J, Juhaszova M, Golovina VA, and Rubin LJ (1998) Molecular basis and function of voltage-gated K^+ channels in pulmonary arterial smooth muscle cells. *Am J Physiol* **274:**L621–L635.

Address correspondence to: Lisa C. Freeman, D.V.M., Ph.D., Department of Anatomy and Physiology, College of Veterinary Medicine, Kansas State University, 228 Coles Hall, Manhattan, KS 66506-5802. E-mail: Freeman@vet.ksu.edu